BACTERIAL OXIDATION OF BENZENE[1]

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The bacterial oxidation of benzene was first reported by Söhngen (1913). Since that time there has been little information added regarding the mechanism by which bacteria attack the intact benzene molecule, although the oxidation of benzene by mammalian tissues has received some attention (Porteus and Williams, 1949). On the other hand, the bacterial oxidation of related aromatic compounds, such as phenol, benzoic acid, naphthalene, and anthracene, has been investigated in some detail. A well-defined pathway of the bacterial degradation of the aromatic nucleus was proposed by Stanier (1950), and several workers, including Murphy and Stone (1955) and Rogoff and Wender (1957, 1959), have studied the breakdown of polycyclic aromatic compounds. The purpose of the work reported here was to study the first steps of benzene oxidation and determine at what points its oxidation products might coincide with the known reactions of the so-called aromatic pathway.

MATERIALS AND METHODS

Organisms capable of utilizing benzene were isolated from soil by an enrichment culture technique in 500-ml Erlenmeyer flasks containing 50 ml medium and 0.1 g benzene, and incubated at 30 C on a reciprocating shaker. The isolation medium had the following composition: NH_4NO_3, 0.4 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $MnCl_2 \cdot 4H_2O$, 0.02 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $CaCO_3$, 0.2 g; tap water, 100 ml; pH 7.0.

Isolation of the benzene-decomposing organisms was accomplished by streaking on nutrient agar and transferring individual colonies back to benzene-mineral salts medium within 24 hr. Purity of cultures was controlled by at least three consecutive transfers through benzene-mineral salts broth to nutrient agar streak plates. For growth and chemical studies a simplified medium was used containing: $NaNH_4HPO_4$, 0.15 g;

KH_2PO_4, 0.10 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; trace $FeCl_2$; and 100 ml tap water, pH 6.8 to 7.0, plus 0.1 g benzene added just prior to inoculation. This medium was free from precipitate and permitted turbidity readings.

Manometric studies were conducted using standard techniques (Umbreit, Burris, and Stauffer, 1957). Cell suspensions were diluted 1:20 on a wet weight basis in 0.02 M phosphate buffer, pH 7.0. Benzene-grown cells were harvested after 24 to 36 hr incubation and nutrient broth-grown cells after 18 hr incubation. When benzene was used as a substrate, it was equilibrated for 5 min in the main flask with the cell suspension in the side arm.

The addition of benzene to the Warburg flasks was accomplished by adsorbing it on dry silica gel. The approximate quantity of benzene added could be determined by weighing the silica gel before and after adsorption. Other substrates were added in the amount of 5 μmoles per flask. Flask contents included 1.0 ml cell suspension, 1.2 ml 0.02 M phosphate buffer, pH 7.0, 0.2 ml 20% KOH, filter paper strip in center well, and 0.5 ml substrate in side arm.

Cell-free extracts were made by grinding 1:20 suspensions of cells in 10 times the wet cell weight of glass beads (type 110 Superbrite glass beads, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota). The suspension was centrifuged for 10 min at approximately $1,000 \times g$. The supernatant was retained and the beads were mixed in a volume of 0.02 M phosphate buffer equal to the original. The suspension was centrifuged as before and then the combined supernatant fractions were centrifuged for 30 min at $15,000 \times g$. Extracts were also made with 1:20 wet cell suspensions using a Raytheon 9 kc sonic oscillator operated for 15 min. The resulting suspension was centrifuged at $1,000 \times g$ for 30 min.

Spectrophotometric studies were made with a Warren Spectracord (Warren Electronics, Inc., Bound Brook, New Jersey), using a double beam of light and balancing with 0.02 M phosphate buf-

fer in both compartments. The spectrum was scanned at 2-min intervals between 220 to 360 mμ until the reaction ceased. The test cuvette contained 0.01 ml cell extract, 0.1 μM catechol, and 3 ml 0.02 M phosphate buffer and was compared with spectra on both the extract and catechol in buffer as controls.

Both cells and cell extracts were tested for the ability to dehydrogenate catechol using standard Thunberg techniques (Umbreit et al., 1957). The tube contained 1.0 ml cell preparation, 0.1 ml 0.1 M methylene blue, and 1.1 ml 0.02 M phosphate buffer; 0.5 ml of a 0.1% solution of catechol was placed in the side arm. Controls containing methylene blue, catechol, and buffer, and methylene blue plus cell preparation were employed.

Ether extraction was conducted on 800-ml quantities of media that showed a strongly positive catechol test (Evans, 1947). The material was placed in the main body of a Kutscher-Steudel extractor and operated for 24 hr using approximately 40 ml ether. The resulting ether solution was concentrated by evaporation to approximately 20 ml. The Rothera reaction (Hawk and Bergeim, 1937) was used to test for β-ketoadipic acid on growing cultures and on large scale resting cell suspensions.

Paper chromatographic studies on catechol were performed on Whatman no. 1 filter paper by spotting 0.2-ml samples of ether extract 1 cm from the origin. Strips (12 by 22 cm) were developed in the solvent system, benzene-acetic acid-water in the ratio 2:2:1, until the solvent had ascended 20 cm, and were air-dried. The papers were treated according to the method of Roux (1951). Succinic acid was identified using a paper chromatographic method (Block, Durrum, and Zweig, 1958). The solvent system of phenol-water-formic acid in the ratio 75:25:1 was used. A control spot containing 5 μmoles succinic acid and a test spot of 1.0 ml culture medium at pH 5.5 were allowed to ascend 50 cm on a strip of Whatman no. 1 filter paper. The papers were air-dried and the color was developed using chlorophenol red (0.04% solution in ethanol, pH 7.0).

The benzene used in these studies was Phillips Petroleum Company (Bartlesville, Oklahoma) Pure Grade, at least 99 mole per cent pure, and was obtained through the courtesy of Dr. J. A. Dixon of the Department of Chemistry, The Pennsylvania State University. The catechol was obtained from the Fisher Scientific Company,

Pittsburgh, Pennsylvania, and the β-ketoadipic acid from Sigma Chemical Company, St. Louis, Missouri. Isolation of o-benzoquinone as dianilino-o-benzoquinone was accomplished by the addition of 1.0 ml aniline to the culture medium. The material was extracted with ether and recrystallized from petroleum ether.

<center>RESULTS</center>

Description and growth characteristics of organisms. Two microorganisms capable of oxidizing benzene as a sole source of carbon were selected from a group of soil isolates. One organism was a gram-positive nonsporeforming rod which broke up into shorter rods and coccal forms. It was nonacid-fast, nonmotile, and formed cream-colored colonies at 30 C on nutrient agar that became orange after 3 days; white filaments could be observed growing from the edges of the colony after 2 weeks. After 7 days of incubation, this culture gave an alkaline reaction on litmus milk and on lactose, maltose, salicin, and sucrose broths; it utilized citrate and reduced nitrate. Tests for reactions on glucose, arabinose, and mannitol; indole production; methyl red; gelatin liquefaction; and H$_2$S formation were negative. On the basis of morphological appearance and physiological tests, the organism was characterized as a strain of *Mycobacterium rhodochrous* (Gordon and Mihme, 1957).

The second organism was a gram-negative rod, occurring singly and in pairs, forming a yellow-green pigment that fluoresced blue under ultra-violet light. The cell was motile with a single polar flagellum and formed a round or amoeboid translucent colony with green, water-soluble pigment at 30 C on nutrient agar. Reactions on the usual differential media with the exception of gelatin liquefaction corresponded closely to the description of *Pseudomonas aeruginosa* (Breed, Murray, and Smith, 1957). Both organisms showed a typical growth pattern in benzene-mineral salts broth, reaching populations of about 10^9 cells/ml within 48 hr.

Manometric studies. Manometric studies with benzene were made using both organisms (Figs. 1 and 2). With cells of *P. aeruginosa* grown on benzene, sufficient oxygen uptake was observed to account for the oxidation of nearly 90% of the benzene to carbon dioxide and water (Table 1). Glucose oxidation was used as a standard of cellular activity. The oxidation rate for cells of *M.*

TABLE 1

Oxidation of benzene and catechol

	Oxygen Uptake		
	Observed	Theoretical	Oxidation
	μl	μl	%
Pseudomonas aeruginosa			
2.4 μM Benzene	359	403	89
5.0 μM Catechol	698	728	96
Mycobacterium rhodochrous			
3.2 μM Benzene	291	538	54
5.0 μM Catechol	340	728	47

Fig. 2. Oxygen consumed by cells of *Mycobacterium rhodochrous* grown on benzene-mineral salts medium. Conditions as in Fig. 1.
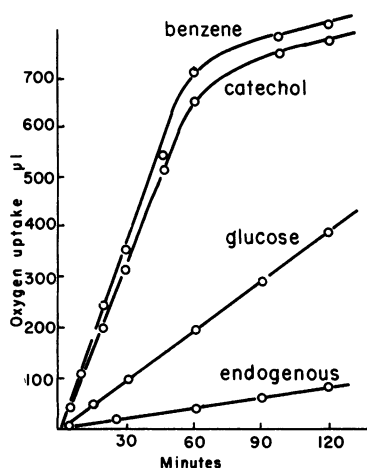

Fig. 1. Oxygen consumed by cells of *Pseudomonas aeruginosa* grown on benzene-mineral salts medium. Temperature 30 C; 5 μmoles substrate in 2. 7 ml 0.016 M phosphate buffer, pH 7.0.

Fig. 3. Oxygen consumed by cells of *Pseudomonas aeruginosa* grown on nutrient medium. Conditions as in Fig. 1.

rhodochrous was somewhat lower than for the pseudomonad on most substrates used. Organisms grown on benzene also oxidized catechol rapidly (Figs. 1 and 2) with sufficient oxygen uptake to account for 96% oxidation to carbon dioxide and water in the case of *P. aeruginosa*. Figures for the oxidation of catechol by both organisms are given in Table 1.

When grown in a nutrient broth medium, both organisms failed to show significant activity on benzene in Warburg studies. With catechol as substrate, however, adaptation (Stanier, 1947) followed by active oxidation could be observed after a lag varying from 20 to 40 min. Typical results using *P. aeruginosa* are shown in Fig. 3. A similar pattern was obtained with *M. rhodochrous*.
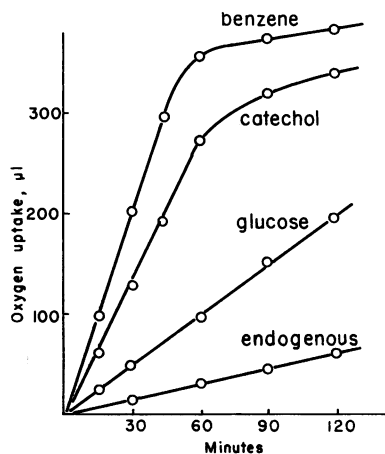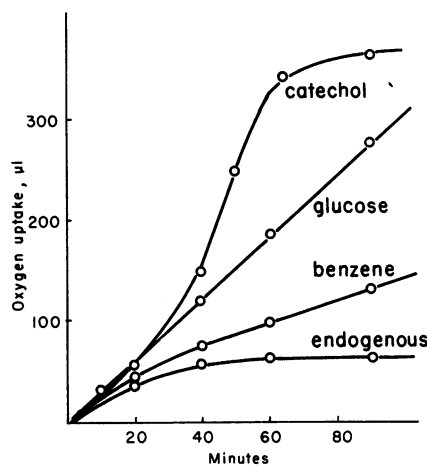
Identification of intermediates. Freshly isolated cultures of *P. aeruginosa* grown in benzene-mineral salts broth were observed to give a positive catechol test after 24 to 48 hr incubation. This property decreased upon subsequent passes of the culture through defined media. Results of paper chromatograms made using ether extracts from media giving a strongly positive catechol test showed identical R_F values (0.41) and the same colors with Roux's reagent and ultraviolet light as the catechol control. Manometric studies on catechol using extracts from pseudomonad cells gave sufficient oxygen uptake to account for the
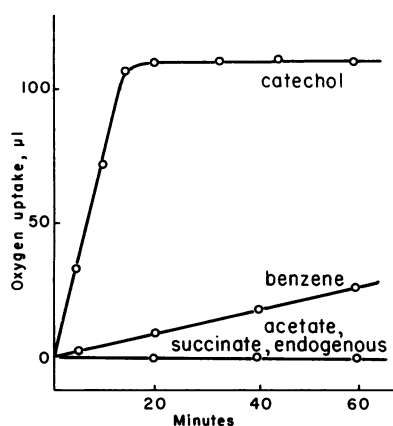
Fig. 4. Oxygen consumed by sonic extracts of *Pseudomonas aeruginosa* grown on benzene-mineral salts medium. Conditions as in Fig. 1.

addition of 1 molecule oxygen per molecule of catechol (Fig. 4). Rothera tests made at the cessation of oxygen uptake were negative, indicating that β-ketoadipic acid was not the final product. Spectrophotometric studies on catechol using cell extracts indicated a continual shift from the 274 mμ peak of catechol toward shorter wavelengths during the first 15 to 20 min of the reaction accompanied by a strong increase in intensity. Solutions diluted 1:100 showed that the product had an absorption maximum at 258 mμ. This figure is in agreement with the reported peak for *cis-cis* muconic acid (Evans et al., 1951).

Evidence that β-ketoadipic acid was an intermediate in the oxidation of benzene was difficult to obtain. Cultures grown in benzene-mineral salts medium did not accumulate sufficient quantities of the compound to give a positive Rothera reaction. When cell-free extracts were used to oxidize catechol in Warburg flasks, *cis-cis* muconic acid appeared to be the end product. Some indication of β-ketoadipic acid was found when intact cells were shaken with an excess of catechol for an extended period of time. A positive Rothera reaction was observed with *P. aeruginosa* after 8 hr incubation at 30 C and with *M. rhodochrous* after 24 hr. Manometric studies using cells in the presence of β-ketoadipic acid showed an oxygen uptake with *P. aeruginosa* of 180 μl oxygen in 90 min. Suspensions of *M. rhodochrous* did not oxidize β-ketoadipic acid at an appreciable rate; this finding is compatible with the results of Gale (1952a, b) using other mycobacteria.

A drop in pH during growth was shown by both organisms in buffered benzene media suggesting the formation of organic acids. As the Rothera and catechol tests were both negative it was assumed that neither β-ketoadipic nor catechol were present in the acidic media in significant amounts. Likewise, spectrophotometric analyses on the media failed to detect the presence of *cis-cis* muconic acid. Chromatographic procedures detected only one acid present in appreciable quantities; the R_F value and acidic properties of this compound corresponded to succinic acid.

An occasional flask of pseudomonad culture growing on benzene turned black. These cultures gave positive catechol tests. The addition of 1 ml aniline to the medium caused a reddish haze to appear. Petroleum ether extraction led to the eventual crystallization of red needles which melted at 190 to 193 C. Appearance and melting point corresponded to dianilino-*o*-benzoquinone which is reported to melt at 193 C (Evans, 1947). If *o*-benzoquinone were an intermediate in catechol oxidation, then the presence of a catechol dehydrogenase would be suspected. Standard Thunberg techniques using both whole cells and cell-free preparations of pseudomonas failed to demonstrate the reduction of methylene blue in the presence of catechol.

Phenol has been implicated as a metabolite of benzene in rabbits (Porteus and Williams, 1949). The possibility that phenol might be an intermediate in the bacterial oxidation of benzene was therefore examined. Cultures were made using 0.1 g and 0.05 g phenol in 50 ml mineral salts broth. Pseudomonas failed to grow on any quantity of phenol tried. The mycobacterium cultures became turbid after a 3- to 5-day lag.

Manometric studies on phenol were made with benzene-grown cells of both *P. aeruginosa* and *M. rhodochrous*. No oxidation of phenol occurred with *P. aeruginosa*; however, some oxidation of phenol did take place with *M. rhodochrous*, the rate increasing with time. Phenol thus failed to meet the criteria of an intermediate since it was not oxidized as rapidly as the parent substance by *M. rhodochrous* and no oxidation or growth could be observed with *P. aeruginosa*.

DISCUSSION

Although several different organisms were isolated from soil enrichment cultures on benzene, only two were studied in detail—a pseudomonas
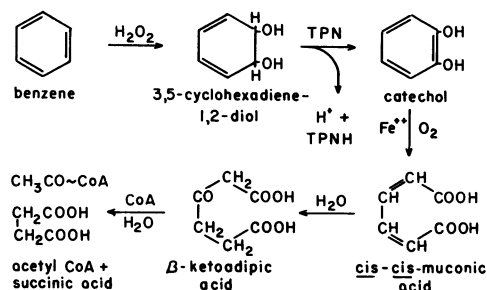
benzene　　　3,5-cyclohexadiene-　　　catechol
1,2-diol　　H⁺ +
TPNH　　Fe⁺⁺ | O₂

CH₃CO~CoA
CH₂COOH
CH₂COOH

acetyl CoA +
succinic acid

β-ketoadipic
acid

cis-cis-muconic
acid

Fig. 5. Proposed pathway of benzene oxidation.

and a mycobacterium isolate. The designation of the former as *Pseudomonas aeruginosa* is somewhat arbitrary and can be questioned if a strict description of this species is adhered to. The chief difference between our organism and the classical description of *P. aeruginosa* (Breed et al., 1957) is that the former does not liquefy gelatin. In other important characters such as colony morphology, formation of yellow-green pigment, polar flagellation, and fermentation characteristics on sugars and litmus milk, the organism closely resembles the type species of this genus. Since these organisms are widespread in the soil and can be selected by enrichment techniques with the ability to attack almost any organic compound, we do not believe that the ability to attack hydrocarbons has significance as a major differential character.

The bacterial oxidation of benzene appears to be confined to strains of those species which are capable of forming enzymes of the so-called "aromatic pathway." The first steps in the oxidation of benzene and other aromatic compounds are apparently unique and can be carried out only by a limited number of strains. In the case of benzene the enzymes involved are not constitutive, as removal of the organisms from benzene media resulted in a loss of ability to oxidize benzene. If the enzymes are adaptive, the conditions necessary for the induction of the enzymes are not met by usual procedures. The ability to oxidize benzene is apparently not due to mutation; growth of equal numbers of organisms on solid defined media and nutrient agar demonstrated that essentially all the organisms were capable of oxidizing benzene. Organisms removed from benzene media and incapable of showing increasing oxygen uptake in the presence of benzene are nevertheless capable of growth in benzene-mineral salts media after a lag of several days. The best explanation

available in terms of present knowledge is that adaptation to benzene must be a slow process.

The usual method of studying the metabolic pathway of a compound is to determine what intermediates in the process accumulate and subsequently disappear from the medium. The rate of production of such compounds in the case of cultures growing on benzene was lower than the demands for cellular growth, and extensive accumulation of intermediates did not occur. Thus other techniques were relied upon for the main body of evidence. Based on the results of the present investigation and upon hypotheses explained in succeeding paragraphs, a proposed mechanism of benzene oxidation is represented in Fig. 5.

The initial attack of the benzene ring has not yet been elucidated. The enzyme proved to be extremely labile as have other similar enzymes (Hayaishi and Stanier, 1951) and the inability to secure the enzyme in cell-free preparations made a study of its function difficult. In addition, the intermediate which may be produced, 3,5-cyclohexadiene-1,2-diol, was unavailable. The enzyme proposed for its dehydrogenation to catechol was therefore not studied. Mammalian dehydrogenation of 3,5-cyclohexadiene-1,2-diol has been shown (Ayenger et al., 1958) and a similar intermediate in the bacterial oxidation of naphthalene, 1,2-dihydronaphthalene-1,2-diol, has been isolated (Young, 1947), suggesting that 3,5-cyclohexadiene-1,2-diol is a likely intermediate in the bacterial oxidation of benzene. The bacterial oxidation of benzene follows the same general metabolic pathway that has been found in bacteria for other aromatic compounds such as phenol, benzoic acid, and naphthalene. The initial reactions merge after several steps into a common route.

From the data now available, the bacterial oxidation of benzene is seen to differ from mammalian oxidation. Compounds that have been isolated from rabbit urine including phenol, catechol, and *trans-trans* muconic acid indicate that more than one route of degradation is available in mammalian tissue. However, the two different bacterial cultures isolated in this study were each able to oxidize benzene completely to carbon dioxide and water by what appeared to be the same general pathway.

SUMMARY

Two organisms, a strain of a *Pseudomonas* species and one of a *Mycobacterium* species, capable

of oxidizing benzene as a sole carbon source, were isolated from soil by an enrichment culture technique. Both organisms oxidized benzene manometrically with sufficient oxygen uptake to account for oxidation to carbon dioxide and water. A metabolic pathway for the breakdown of benzene via 3,5-cyclohexadiene-1,2-diol and catechol is proposed. Phenol and o-benzoquinone are not probable intermediates in the oxidation of benzene by these organisms.

REFERENCES

AYENGAR, P. K., O. HAYAISHI, M. NAKAJIMA, AND I. TOMITA 1958 Enzymatic aromatization of 3,5-cyclohexadiene-1,2-diol. Abstracts, 133rd Meeting Am. Chem. Soc., San Francisco, 29c.

BLOCK, R. J., E. L. DURRUM, AND G. ZWEIG 1958 *A manual of paper chromatography and paper electrophoresis*, 2nd ed. Academic Press, Inc., New York.

BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH 1957 *Bergey's manual of determinative bacteriology*, 7th ed. The Williams & Wilkins Co., Baltimore.

EVANS, W. C. 1947 Oxidation of phenol and benzoic acid by some soil bacteria. Biochem. J., **41**, 373–382.

EVANS, W. C., B. S. W. SMITH, R. P. LINSTEAD, AND J. A. ELVIDGE 1951 Chemistry of the oxidative metabolism of certain aromatic compounds by microorganisms. Nature, **168**, 772–775.

GALE, G. R. 1952a The oxidation of benzoic acid by mycobacteria. I. Metabolic pathways in *Mycobacterium tuberculosis*, *Mycobacterium butyricum*, and *Mycobacterium phlei*. J. Bacteriol., **63**, 273–278.

GALE, G. R. 1952b The oxidation of benzoic acid by mycobacteria. II. The metabolism of postulated intermediates in the benzoate oxidation chain by four avirulent and two virulent organisms. J. Bacteriol., **64**, 131–135.

GORDON, R. E. J., AND M. MIHME 1957 A comparative study of some strains received as Nocardiae. J. Bacteriol., **73**, 15–27.

HAWK, P. B., AND O. BERGEIM 1937 *Practical physiological chemistry*, 11th ed. The Blakiston Co., Philadelphia.

HAYAISHI, O., AND R. Y. STANIER 1951 The bacterial oxidation of tryptophan. III. Enzymatic activities of cell-free extracts from bacteria employing the aromatic pathway. J. Bacteriol., **59**, 691–709.

MURPHY, J. F., AND R. W. STONE 1955 The bacterial dissimilation of naphthalene. Can. J. Microbiol., **1**, 579–588.

PORTEUS, J. W., AND R. T. WILLIAMS 1949 Studies in detoxication. 20. The metabolism of benzene. 2. The isolation of phenol, catechol, quinol and hydroxyquinol from the ethereal sulphate fraction of the urine of rabbits receiving benzene orally. Biochem. J., **44**, 56–61.

ROGOFF, M. H., AND I. WENDER 1957 3-Hydroxy-2-naphthoic acid as an intermediate in bacterial dissimilation of anthracene. J. Bacteriol., **74**, 108–109.

ROGOFF, M. H., AND I. WENDER 1959 Methylnaphthalene oxidations by pseudomonads. J. Bacteriol., **77**, 783–788.

ROUX, D. B. 1951 Colour reagents for the paper chromatography of di- and trihydroxyphenols. Nature, **168**, 1041–1042.

SÖHNGEN, N. L. 1913 Benzin, Petroleum, Paraffinol and Paraffin als Kohlenstoff und Energiequelle fur Mikroben. Centr. Bakteriol. Parasitenk., Abt. II, **37**, 595–609.

STANIER, R. Y. 1947 Simultaneous adaptation: a new technique for the study of metabolic pathways. J. Bacteriol., **54**, 339–348.

STANIER, R. Y. 1950 Problems of bacterial oxidative metabolism. Bacteriol. Rev., **14**, 179–191.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER 1957 *Manometric techniques*. Burgess Publishing Co., Minneapolis.

YOUNG, L. 1947 The metabolic conversion of naphthalene to 1,2-dihydronaphthalene-1,2-diol. Biochem. J., **41**, 417–422.